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Signaling Mechanisms Regulating Self-Renewal and Differentiation of Pluripotent Embryonic Stem Cells

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Key Words

Embryonic stem cells · Leukemia inhibitory factor · gp130 · STAT3 · Extracellular regulated kinase

Abstract

An ability to propagate pluripotent embryonic cells in culture is the foundation both for defined germline modification in experimental rodents and for future possibilities for broad-based cellular transplantation therapies in humans. Yet, the molecular basis of the self-renewing pluripotent phenotype remains ill-defined. The relationship between factors that influence embryonic stem cell propagation in vitro and mechanisms of stem cell regulation operative in the embryo is also uncertain. In this article we discuss the role of intracellular signalling pathways in the maintenance of pluripotency and induction of differentiation in embryonic stem cell cultures and the mammalian embryo.

Introduction

Mouse embryonic stem (ES) cells are continuous cell lines derived directly from the founder tissue of the embryo, the epiblast [Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997]. They have apparently unlimited proliferative potential and can be propagated clonally with retention of non-transformed phenotype and euploid karyotype. Like the epiblast, ES cells are pluripotent, that is they are capable of multilineage differentiation. In vitro, ES cells can generate extra-embryonic cell types and derivatives of the three primary germ layers, ectoderm, mesoderm and endoderm. Most remarkably, in vivo, ES cells can be reincorporated into normal embryonic development and contribute to all developing cell lineages, including the germ line. ES cells thus represent capture in culture of the normally transient phase of embryonic pluripotency [Smith, 1992].

Abbreviations used in this paper

CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
ERK	extracellular regulated kinase
ES	embryonic stem cells
G-CSFR	granulocyte-colony-stimulating factor receptor
IL-6	interleukin-6
IRS	insulin receptor substrate
JAK	Janus kinase
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinases
OSM	oncostatin M
SH2	Src-homology 2
sIL-6R	soluble IL-6 receptor
STAT	signal transducer and activator of transcription

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The ES cell genome can be manipulated with precision and relative ease [Smith et al., 1995], thereby enabling the generation of mice with defined genetic lesions. ES cell technology has consequently become a central research tool for determination of mammalian gene function and generation of animal models. To date, however, this technology has not been transferred to any species other than mouse despite considerable efforts [Gardner and Brook, 1997]. The barrier to establishing pluripotent ES cells from non-mouse embryos is not understood. Recent reports of primate and human embryo-derived cultures that share some of the properties of mouse ES cells are tantalising therefore [Thomson et al., 1998; Thomson and Marshall, 1998]. Human pluripotent cell lines would open up the possibility of cell therapy. In vitro stem cell differentiation could be used to produce tissue for cell replacement to treat a variety of genetic and acquired human ailments. If this prospect is to be realised, however, the human stem cells must exhibit the full proliferative potential and in vitro differentiation repertoire of mouse ES cells [Smith, 1998]. Furthermore, although progress to date has been dictated largely by empirical approaches, ultimately a rational appreciation of stem cell molecular biology is required to deliver clinical benefits with appropriate safeguards.

We describe here investigations into the signaling mechanisms by which cytokines sustain propagation of pluripotent ES cells. These studies have revealed both positive and negative effectors, suggesting that the decision between self-renewal and commitment is dictated by a balance of conflicting intracellular signals. Further understanding is needed, however, of how these opposing signals are generated and regulated and of the cross-talk between them. Such knowledge will inform and guide efforts to establish ES cell equivalents in other species and in addition may illuminate mechanisms of lineage commitment in the developing mammalian embryo.

Cytokine Dependence of Mouse ES Cells

Mouse ES cell lines are established by picking and expanding stem cell outgrowths derived from the inner cell masses of cultured blastocysts. Initial experimental protocols required that the stem cells were cultured on a monolayer of mouse embryo fibroblasts [Evans and Kaufman, 1981; Martin, 1981]. Without this feeder layer pluripotent cells could not be maintained, suggesting that the fibroblasts acted directly either to promote self-renewal or to suppress differentiation. Subsequent isolation of a soluble glycoprotein that prevents stem cell differentiation established that ES cell self-renewal is dependent on paracrine signal(s) [Smith and Hooper, 1987; Smith et al., 1988]. This factor is the cytokine leukaemia inhibitory factor (LIF) [Gearing et al., 1987; Williams et al., 1988].

LIF is a member of a family of related cytokines including interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin 1 (CT-1) [Taga and Kishimoto, 1997]. These factors have overlapping, pleiotropic effects on a variety of different cell types. In fact, OSM, CNTF and CT-1 can all suppress the differentiation of ES cells [Conover et al., 1993; Yoshida et al., 1994; Pennica et al., 1995]. In contrast, ES cells cannot be maintained by IL-6 or IL-11 since the specific receptors for these cytokines are not expressed on ES cells. However, if a soluble form of the IL-6 receptor is provided, IL-6 will support stem cell self-renewal [Nichols et al., 1994; Yoshida et al., 1994].

The basis for the shared activity of these cytokines is clear when the molecular structure of their receptor complexes is considered. LIF, OSM and CT-1 bind directly to a heterodimeric receptor complex containing two transmembrane glycoproteins, gp130 and LIFR [reviewed in Taga and Kishimoto, 1997; Heinrich et al., 1998] (fig. 1). CNTF also engages the heterodimer, but the interaction is mediated via binding to a GPi-anchored ligand binding protein, CNTFR. IL-6 acts independently of LIFR, through a hexameric receptor complex containing a homodimer of gp130 complexed with two ligand-bound IL-6 receptor modules. Thus, engagement of the gp130 receptor subunit is the common requirement for promotion of ES cell self-renewal by the LIF-related cytokines.

gp130 is a member of the cytokine receptor superfamily [Bazan, 1990]. In common with the other family members, the intracellular domain of gp130 has no intrinsic kinase activity. This function is provided via the association of Janus kinase (JAK) non-receptor tyrosine kinases with the membrane proximal, proline-rich, Box 1 region of gp130 [Darnell et al., 1994]. Ligand-induced oligomerisation of gp130 brings the associated JAKs into close proximity, causing their cross-phosphorylation and activation. JAKs 1, 2 and Tyk 2, are all reported to associate with gp130 [Lutticken et al., 1994; Stahl et al., 1994]. However, studies performed with mutant cell lines and mice lacking JAK 1 indicate that activity of this kinase in particular may be critical for eliciting downstream signaling events [Guschin et al., 1995; Rodig et al., 1998].

JAK activation is regarded as obligatory for tyrosine phosphorylation of the receptor and other downstream

molecules. Receptor phosphotyrosines form binding sites for specific proteins containing phosphotyrosine binding motifs, such as the Src-homology 2 (SH2) domain. These bound molecules can then serve as further substrates for the JAK kinases. Whilst a degree of signaling specificity may reside in the JAK kinases, results of receptor domain swap experiments demonstrate that many key signals activated by cytokines are determined by the identity of the receptor tyrosine modules and the signal transducers that they recruit [Stahl et al., 1995].

A wide range of downstream effector molecules can be activated via gp130 [reviewed in Heinrich et al., 1998]. These include the signal transducer and activator of transcription (STAT) 1, 3 and 5 transcription factors, the tyrosine phosphatase SHP-2, the insulin receptor substrate (IRS) family proteins, IRS-1, IRS-2 and Gab1, the mitogen-activated protein kinases (MAPK), extracellular regulated kinases (ERK) 1 and 2, phosphoinositol-3 kinase, and the Src family tyrosine kinases, Hck, Btk and Fes. Some of these signals may be cell type-specific and their contribution to eliciting biological effects downstream of gp130 in many cases remains to be determined. It has been clearly established, however, that activation of the STAT and ERK signaling pathways do play essential roles in mediating the biological responses to cytokines acting through gp130. Activation of STAT3 is required for gp130-dependent differentiation of the M1 myeloid cells [Minami et al., 1996; Nakajima et al., 1996] and promotes the differentiation of astrocytes [Bonni et al., 1997]. In the BAF3 pro-B-cell line, STAT3 induces antiapoptotic factors and suppresses antiproliferative signals [Fukada et al., 1996, 1998]. On the other hand, stimulation of the MAPK pathway via gp130 has been shown to promote neurite outgrowth [Ihara et al., 1997], cardiomyocyte survival [Sheng et al., 1997], and BAF3 cell proliferation [Fukada et al., 1996].

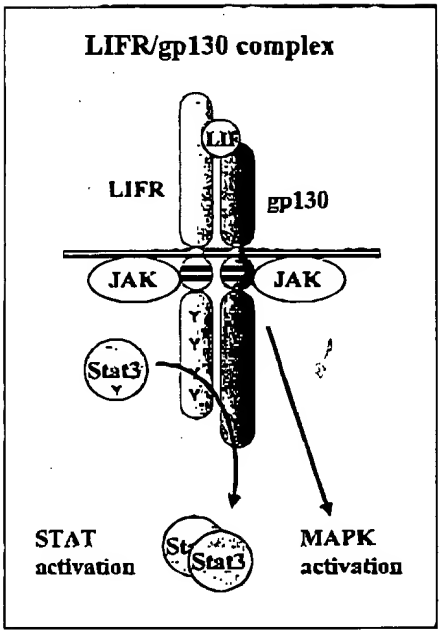

Fig. 1. The LIFR/gp130 signaling complex. LIF binds to LIFR and gp130, thereby bringing together the receptor-associated JAK kinases. Subsequent JAK activation and phosphorylation of receptor tyrosines then lead to stimulation of both the STAT and MAPK signaling pathways.

Activation of STAT3

STAT3 was first identified as a component of the IL-6-induced DNA binding activity, acute phase response factor [Akira et al., 1994; Zhong et al., 1994]. Subsequently, it was shown to be activated by a variety of cytokine receptors, receptor tyrosine kinases and non-receptor tyrosine kinases. Seven mammalian STAT genes have been identified and encode proteins with a similar overall structure [Darnell, 1997]. These proteins contain a highly conserved amino terminus that mediates interactions between activated STAT dimers, a centrally located DNA binding region, an SH2 domain, an adjacent, positionally conserved regulatory tyrosine and a carboxy terminal region containing a transactivation domain (fig. 2). The most striking feature of STATs, the SH2 domain, is not found in any other known family of transcription factors. This enables STATs to link activation of receptors at the cell surface directly with transcriptional events in the nucleus.

The SH2 domain mediates the selective recruitment of STATs to activated receptor complexes. STAT3, for instance, has been shown to bind receptors carrying YpXXQ motif (where X represents any amino acid) [Stahl et al., 1995; Gerhartz et al., 1996]. Receptor-associated STATs are then subject to the attentions of tyrosine kinases, becoming phosphorylated on the conserved tyrosine, immediately adjacent to the SH2 domain [Wen et al., 1995]. Upon dissociation from the receptor, STATs dimerise through reciprocal interactions involving the SH2 domains and phosphotyrosines. Activation of STAT3 is often accompanied by phosphorylation of STAT1, with which it can heterodimerise. The balance of homodimers and heterodimers is receptor- and cell type-dependent and can be influenced by the level of activating

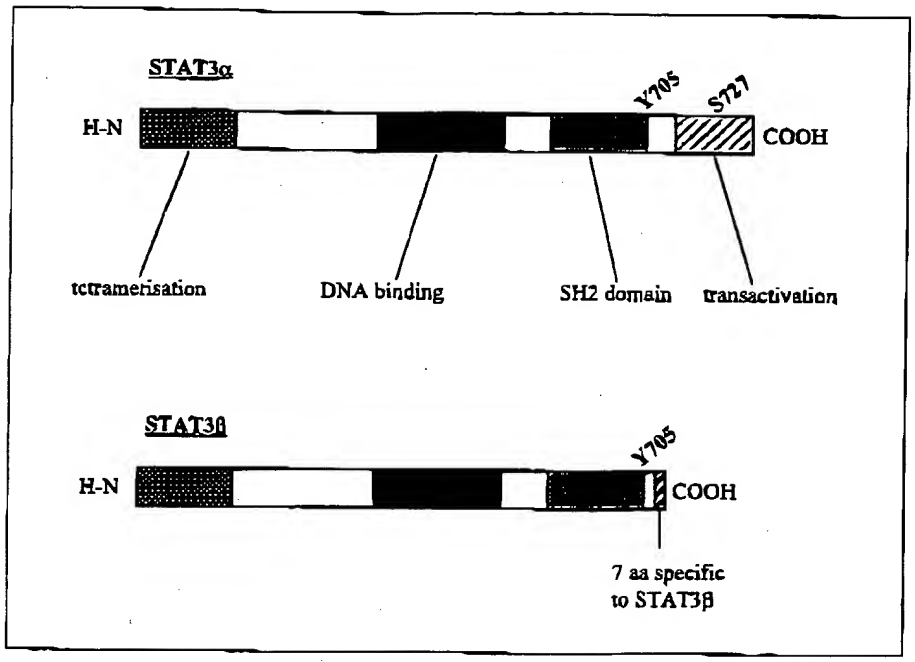

Fig. 2. Functional domains of STAT3.

ligand [Darnell, 1997]. STAT dimers then rapidly translocate into the nucleus, where they bind to DNA and regulate transcription of target genes. All STAT transcription factors (with the exception of STAT2) can bind to variants of the palindromic consensus sequence TTCnnnGAA, presumably reflecting the symmetry of the STAT dimer [Hoey and Schindler, 1998].

STAT3β is a naturally occurring splice variant of the full-length STAT3 (STAT3α) in which the carboxy terminal 55 residues encompassing the transactivation domain are replaced with an unrelated 7-residue peptide [Schaefer et al., 1995] (see fig. 2). The truncated STAT3β protein retains tyrosine 705 and can therefore be activated, forming dimers capable of binding to the STAT DNA recognition site [Schaefer et al., 1997]. Although STAT3β can function as an inhibitor of full-length STAT3, under some circumstances it transactivates STAT3 reporter genes, possibly directly or through an association with the Jun B transcription factor [Schaefer et al., 1995; Sasse et al., 1997; Schaefer et al., 1997]. Interestingly, following cytokine stimulation, the tyrosine phosphorylated form of STAT3β persists for longer than activated STAT3α, indicating that turnover of activated STAT3 is regulated through its carboxy terminus.

The transactivation domain of STAT3α is also subject to ligand-dependent phosphorylation of residue serine 727 [Wen et al., 1995]. A mutant STAT3, lacking S727 exhibits reduced transcriptional activation of STAT-dependent reporter genes and inhibits STAT3-dependent transformation [Wen et al., 1995; Bromberg et al., 1998]. The phosphorylation of S727 may therefore contribute to the transactivation function of STAT3. Interestingly, S727 is situated within a recognised consensus recognition site for MAPK [Chung et al., 1997], indicating that phosphorylation of S727 may represent a direct mechanism for integrating MAPK and STAT signaling pathways. The kinase responsible for serine phosphorylation in vivo has not yet been identified, however.

STAT3 and Regulation of ES Cell Self-Renewal

STAT3 is the principal STAT factor activated in ES cells stimulated with either IL-6 plus soluble IL-6 receptor (sIL-6R) (fig. 3), or with LIF [Hocke et al., 1995]. The majority of STAT DNA binding complexes are comprised of STAT3 homodimers (fig. 3), although low levels of activated STAT1 can also be detected [Hocke et al., 1995; Starr et al., 1997]. On the basis of functional assays in somatic cell lines and in vitro peptide binding experiments, four tyrosines within the cytoplasmic domain of gp130 conforming to the YXXQ STAT3 consensus binding motif represent potential STAT docking sites [Stahl et al., 1995; Gerhartz et al., 1996]. The most membrane proximal site is contained within the conserved box 3 domain (Y126, situated 126 amino acids from the cyto-

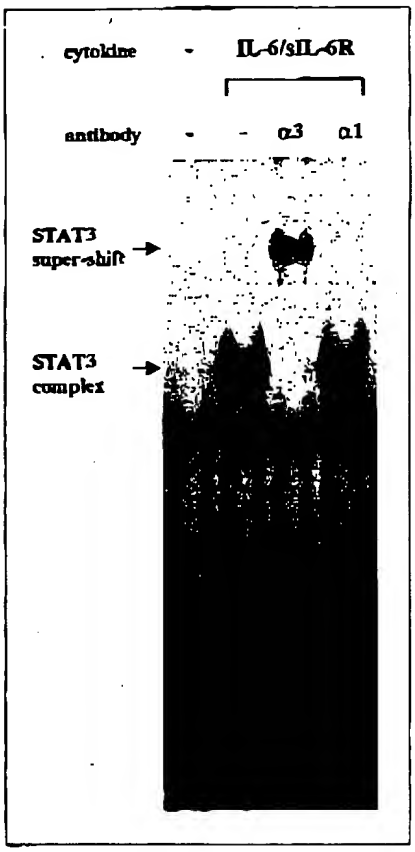

Fig. 3. gp130-dependent activation of STAT3 in ES cells. Nuclear extracts prepared from uninduced ES cells or cells treated with IL-6 plus sIL-6R were incubated with a ^{32}P-labelled oligonucleotide containing a STAT DNA recognition site and fractionated on a non-denaturing polyacrylamide gel. The induced STAT DNA binding complex is composed mainly of STAT3 (homodimers), since it is completely supershifted by antibodies specific for STAT3 (α3), but unaffected by antibodies recognising STAT1 (α1).

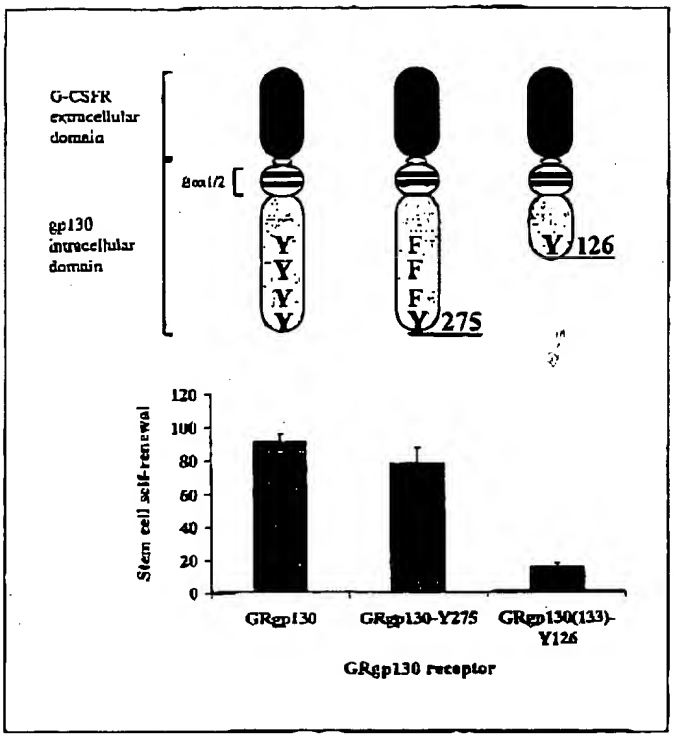

Fig. 4. Amino-terminal STAT3 binding site in gp130 is sufficient for directing ES cell self-renewal. ES cell transfectants expressing G-CSFR/gp130 receptor chimaeras were plated at medium density (5,000 cells per well of a 24-well dish) and cultured for 6 days in medium containing either no cytokine, G-CSF or IL-6 plus sIL-6R. Stem cell self-renewal was measured by assaying stem cell-specific expression of a chromosomal *Oct4-lacZ* reporter gene. The results presented in the graph represent the level of β-galactosidase activity induced by G-CSF, expressed as a percentage of that obtained with IL-6 plus sIL-6R. The data shown is the mean values (±SEM) obtained from three independent transfectants for each chimaeric receptor construct.

plasmic face of the membrane). The next motif is located at 173 amino acids (Y173) and the remaining two are situated adjacent to each other at the very carboxy terminus of the receptor (Y265, Y275).

In order to assess the contribution of STAT3 to ES cell self-renewal, chimaeric cytokine receptors consisting of the extracellular domain of the granulocyte-colony-stimulating factor receptor (G-CSFR) fused to either the wild type or mutated forms of the intracellular domain of gp130 were stably introduced into ES cells. The recipient cell line used in these studies contains a β-galactosidase gene integrated into the genomic locus encoding the essential stem cell-specific transcription factor Oct4 [Nichols et al., 1998]. As a result, expression of the *lacZ* reporter gene is restricted to undifferentiated ES cells and provides a reliable measure of stem cell self-renewal [Dani et al., 1998].

G-CSFR is a cytokine receptor that forms homodimers in response to administration of G-CSF. ES cells do not express the endogenous G-CSFR and no response to G-CSF is evident either biochemically or at the level of self-renewal. This system, therefore, allows the functional analysis of gp130-dependent signals independently from the contribution of endogenous cytokine receptors. In fact, the chimaeric receptor containing the complete cytoplasmic domain of the gp130 receptor is fully capable of directing ES self-renewal [Starr et al., 1997; Niwa et al., 1998], demonstrating that the hybrid molecule reproduces the essential functions of endogenous gp130 in ES cells (fig. 4).

Substitution of all four STAT3 binding tyrosines with phenylalanine within this chimaeric receptor abolishes both activation of STAT3 and the capacity of the receptor to prevent the differentiation of ES cells [Niwa et al., 1998]. Nevertheless, the receptor still engages productively with G-CSF, as is evident by its ability to activate the MAPK pathway. Interestingly, the four YXXQ binding sites on gp130 are not equally effective at activating STAT3. Loss of the two proximal sites, Y126, and Y173, does not appreciably diminish the activation of STAT3 or self-renewal. In contrast, substitution of both Y265 and Y275 with phenylalanine significantly reduces activation of STAT3 and impairs the ability of the receptor to promote ES cell self-renewal. This suggests that in ES cells only the terminal tyrosine motifs of gp130 are fully effective in activating STAT3. This conclusion is supported by the findings that a chimaeric receptor in which only the terminal Y275 STAT3 binding site is intact is capable of blocking the differentiation of ES cells (fig. 4). In comparison, a truncated chimaeric receptor containing only the most proximal YXXQ motif, which is reported to be effective biochemically and biologically in M1 or BAF3 cells [Fukada et al., 1996; Yamanaka et al., 1996], is incapable of delivering a full self-renewal response in ES cells.

The molecular basis for this disparity is not understood. Such differences could be trivial and artifactual: it may be an inherent feature of continual passaging that cells are selected for transformation or increased sensitivity to extracellular signals. Such cells could be particularly adept at rescuing otherwise weak STAT3 binding sites from signaling obscurity. This explanation, however, provides no insight into the presence of four potential STAT3 binding sites in gp130. All four sites are conserved between mouse and man suggesting that there have been selective pressures for retaining their functions and that they play important individual or additive roles under specific circumstances or in particular cell types. Interestingly, the gp130-related cytokine receptor, G-CSFR, expression of which is limited to myeloid cell types, contains only one consensus STAT3 binding site. Multimerisation of the STAT3 docking sites on gp130 may have been instrumental in its recruitment to functions in a wide variety of different cell types, including epiblast cells. One possibility is that differential association of other signaling complexes, for example through recruitment to Y118 (see below), may influence the accessibility of particular YXXQ motifs.

It is also conceivable that the two terminal tyrosines in gp130 have functions in ES cells other than their ability to engage STAT3 and these additional signals are necessary for self-renewal. STAT1 can be activated via gp130 and the terminal two tyrosines have been reported to be particularly important in this process [Gerhartz et al., 1996]. It is unlikely, however, that STAT1 is required for self-renewal since STAT1$^{-/-}$ ES cells can be propagated in a LIF-dependent manner [Durbin et al., 1996].

Inhibition of STAT3 signaling directly, through the use of interfering mutant forms of the transcription factor, provides the best evidence for an essential role of STAT3 in ES cell self-renewal. Expression of STAT3F, a mutant in which the tyrosine 705 required for dimerisation is substituted with phenylalanine, blocks STAT3 activity presumably by either competing for receptor binding sites, or by forming non-functional complexes with endogenous STAT3 [Kaptein et al., 1996; Minami et al., 1996]. Significantly, ES cell transfectants that constitutively express high levels of STAT3F from a stably integrated transgene cannot be propagated [Boeuf et al., 1997; Niwa et al., 1998]. The basis for this observation became apparent by using a polyoma-based episomal expression system, in which transfection efficiencies 100-fold higher than conventional protocols can routinely be attained [Gassmann et al., 1995; Niwa et al., 1998]. Inspection of the resulting ES cell transfectants established that high levels of STAT3F expression cause ES cells to differentiate [Niwa et al., 1998]. Importantly, whereas co-expression of STAT3 with STAT3F restores stem cell self-renewal, overexpression of STAT1 does not, indicating that this closely related STAT family member cannot functionally substitute for STAT3. Furthermore, conditional expression of an integrated, tetracycline-inducible STAT3F transgene also blocks ES cell self-renewal [Niwa et al., 1998]. In conjunction with the results obtained with the receptor chimaeras, these data establish that activation of STAT3 is critical for gp130-dependent self-renewal of ES cells.

Whereas the evidence for a role of STAT3 in ES cells is compelling, the importance of gp130 signaling in promoting stem cell self-renewal in the early embryo is unclear. Although LIF, LIFR and more significantly, gp130, are all expressed appropriately within the blastocyst [Nichols et al., 1996], deletion of any of these individual components through homologous recombination does not prevent formation of the epiblast, nor appreciably affect egg cylinder morphogenesis or gastrulation [Stewart et al., 1992; Li et al., 1995; Yoshida et al., 1996]. STAT3$^{-/-}$ embryos, in contrast, are reported to die early in development, at about embryonic day 7.5 [Takeda et al., 1997]. At this stage the embryos have formed an egg cylinder but are reported to be unable to progress through gastrulation. In

situ hybridisation experiments indicate that STAT3 transcription is first expressed in the visceral endoderm [Takeda et al., 1997]. On this basis, it was proposed that the early developmental failure is caused by a defect in the formation of extraembryonic tissues [Takeda et al., 1997]. However, when STAT3$^{-/-}$ blastocysts are cultured in vitro the inner cell mass does not thrive, consistent with a requirement for STAT3 in the maintenance of ES cells. The discrepancy between the phenotype of the gp130 and STAT3 null embryos and the importance of gp130 signaling in vitro, however, does necessitate careful reconsideration of the relationship between epiblast stem cells in vivo and ES cells. If STAT3 is dispensable for expansion of the epiblast it is likely that other signals operate in vivo. The report of a gp130-independent factor secreted by extraembryonic cells, that can suppress differentiation of ES cells but does not activate STAT3, supports the existence of alternative self-renewal pathways [Dani et al., 1998].

Although STAT3 is not required for the development of the epiblast, it could function in an adaptive mechanism for maintaining stem cell pluripotency when early embryonic development is perturbed. Such a situation occurs naturally in rodents when a lactating female conceives while still nursing. Under these circumstances there is a delay in implantation of the blastocysts, termed diapause. This can also be induced experimentally by ovariectomising a pregnant mouse shortly after conception [Hogan et al., 1994]. In this condition, implantation of blastocysts in the uterus wall is blocked. The blastocysts are viable, however, remaining in a state of developmental arrest within the uterus for up to 4 weeks. When transplanted into the uterus of a surrogate mouse, or following administration of oestrogen, they can implant and reinitiate normal development. Aside from a slight increase in the number of cells in the epiblast, little is known about maintenance of stem cells during delayed implantation. Interestingly, however, implantation delay appears to enhance the frequency of ES cell derivation [Evans and Kaufman, 1981; Gardner and Brook, 1997]. STAT3 may therefore contribute to maintaining the pluripotency of stem cells during diapause, which would provide a rationale for its role in promoting ES cell self-renewal in vitro.

gp130-Dependent Activation of SHP-2 and ERK Signaling

The other major class of signals shown to be activated downstream of gp130 are MAP kinases. ERKs 1 and 2 are activated in ES cells stimulated with LIF or IL-6 plus sIL-6R [Ernst et al., 1996; Boeuf et al., 1997] (fig. 5). These signal transducers attract interest because of their well-documented functions in regulating the proliferation and survival of somatic cell types [Lloyd, 1998]. A possible role for this pathway in ES self- renewal has been implied by observations that expression of upstream activators of ERKs, either constitutively activated forms of Hck, a Src family kinase that may be upstream of Ras, or a hyperactive form of Ras itself, reduced the requirement of ES cells for LIF [Ernst et al., 1994]. This issue is contentious, however, since over-expression of, or constitutive activation of, proto-oncogenic tyrosine kinases can have unforeseen regulatory consequences. There is evidence that Src itself can also promote ES cell self-renewal [Boulter et al., 1991], and this could be explained through its established ability to activate STAT3 [Yu et al., 1995; Cao et al., 1996]. Furthermore, expression of a constitutively active form of Ras in ES cells is reported to direct visceral endoderm differentiation [Cheng et al., 1998].

One established route for receptor-mediated activation of ERKs in somatic cells is through the cytosolic protein tyrosine phosphatase SHP-2. This ubiquitously expressed phosphatase is homologous to the *Drosophila* protein corkscrew and closely related to the mammalian haematopoietic cell phosphatase SHP-1 [reviewed in Neel and Tonks, 1997; Van Vactor et al., 1998]. The common

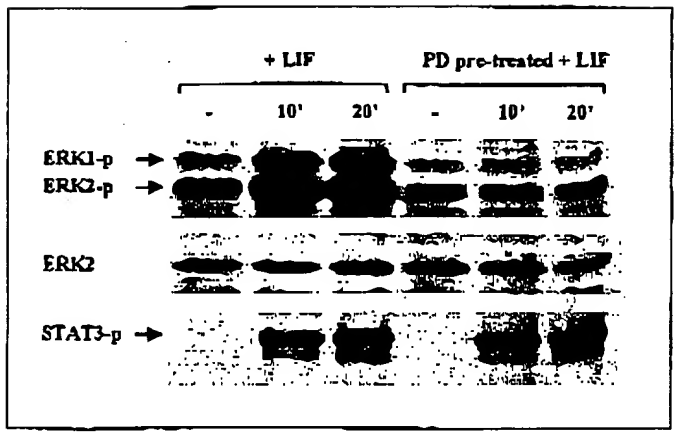

Fig. 5. LIF-dependent ERK activation in ES cells. ES cells either pretreated overnight with the MEK inhibitor PD098059 (PD, 50 μM), or untreated, were stimulated with LIF for 0, 10 and 20 min and analysed by Western blotting. Activation of ERKs 1 and 2 (recognised by a phospho-ERK-specific antibody) is rapidly induced after treatment with LIF. Significantly, the pretreatment of ES cells with PD098059 blocks ERK activation but does not affect the level of ERK protein or impair STAT3 activation (recognised by a phospho-STAT3-specific antibody).

structural features of this family of proteins are two tandemly arranged SH2 domains in the amino terminus and a catalytic domain within the carboxy terminus. The SH2 domains recruit SHP-2 to activated receptors and potential substrates. In addition, engagement of the amino-terminal SH2 motif, by preventing its intramolecular association with the phosphatase domain, increases the catalytic activity of SHP-2 [Hof et al., 1998].

SHP-2 is recruited to gp130 through a single phosphorylated tyrosine, located 118 amino acids from the membrane within the box 3 region [Stahl et al., 1995]. Mutation of Y118 both prevents binding of SHP-2 and blocks the activation of ERKs via gp130 [Fukada et al., 1996]. Two alternative, but not necessarily exclusive, mechanisms may explain how SHP-2 couples to the ERK pathway. Upon recruitment, SHP-2 becomes phosphorylated on two tyrosines situated close to carboxy terminus [Bennett et al., 1994; Fukada et al., 1996]. One of these, Y542, constitutes a binding site for the adaptor protein Grb2. This interaction provides a relatively direct mechanism by which SHP-2 can be coupled through Grb2 and Sos to Ras, thereby activating the ERK signaling pathway [Li et al., 1994]. Alternatively, activation of ERKs can occur through the association of SHP-2 with a potential substrate, Gab1, an IRS-like multifunctional adaptor protein [Holgado-Madruga et al., 1996; Takahashi-Tezuka et al., 1998]. Genetic and biochemical evidence in *Drosophila* identifies a Gab1-related protein, Dos, as a necessary substrate for signaling through the corkscrew protein [Herbst et al., 1996; Raabe et al., 1996]. Furthermore, experiments performed with mammalian cells suggest that Gab1 and SHP-2 are recruited to gp130, forming a complex with and activating phosphoinositol-3-kinase, which in turn stimulates ERK signaling via a Ras-dependent pathway [Takahashi-Tezuka et al., 1998].

In addition to its documented role as positive effector, recent studies suggests that SHP-2 can also have a negative role in signaling. Indeed, this was hinted at by its homology to SHP-1, an established negative regulator, and its association with CTL-4, an inhibitor of T cell receptor activity [Marengere et al., 1996]. Direct evidence for a negative role has now been provided by studies of gp130 signaling. Elimination of the SHP-2 binding site in gp130 or overexpression of catalytically inactive SHP-2 mutants results in enhanced activation of JAK kinases, prolonged phosphorylation of gp130 and STAT3, and increased transcription of STAT3 target genes [Symes et al., 1997; Kim et al., 1998; Servidei et al., 1998]. These results have been taken to indicate that SHP-2 may directly dephosphorylate JAKs or the receptor. However, the reported antagonism of STAT3 activity by ERK signaling suggests that enhanced STAT3 activity associated with the absence of SHP-2 function could also result from the loss of signals downstream from the phosphatase [Bonni et al., 1997; Ihara et al., 1997].

Role of SHP-2/ERK Signaling in ES Cell Self-Renewal

SHP-2, a Gab1-related protein and ERKs 1 and 2 are activated in ES cells stimulated with IL-6 plus sIL-6R (fig. 5) [Burdon et al., 1999]. To examine the role of this pathway, a chimaeric G-CSFR/gp130 receptor in which the Y118 SHP-2 binding site is mutated to phenylalanine was stably introduced into ES cells. This receptor does not engage SHP-2, yet is fully capable of directing the propagation of ES cells [Burdon et al., 1999]. Indeed, the mutant receptor exhibits increased signaling activity as evident by prolonged activation of STAT3 and enhanced ES cell self-renewal.

This lack of a requirement for SHP-2 activity is supported by results obtained from ES cells in which both copies of the phosphatase gene have been mutated by homologous recombination [Saxton et al., 1997; Qu and Feng, 1998]. The targeted deletion eliminates 65 amino acids from the amino terminal SH2 domain and increases the constitutive activity of the phosphatase [Qu et al., 1997]. However, the mutant SHP-2 protein ($SHP\text{-}2^{\Delta 46-110}$) is expressed at reduced levels and without a functional amino terminal SH2 domain would not be expected to interact effectively with many of its target proteins. This contention is supported by the reported deregulation of signaling via several different types of receptor in $SHP\text{-}2^{\Delta 46-110}$ cells [Saxton et al., 1997]. Nevertheless, the mutant ES cells are viable and in fact exhibit increased sensitivity to LIF [Qu and Feng, 1998], consistent with the notion that SHP-2 acts primarily as negative regulator of self-renewal signaling in ES cells [Burdon et al., 1999].

The absence of an essential requirement for SHP-2 function in ES cells implies that ERK signaling does not contribute directly to stem cell self-renewal. Indeed, minimal activation of ERKs is detected following stimulation through the mutant G-CSFR/gp130 Y118F receptor [Burdon et al., 1999]. Furthermore treatment of ES cells with blocking concentrations of PD098059, an inhibitor of the MEK enzymes that activate ERKs (fig. 5), does not impair the propagation of ES cells. In fact, ES cells cultured in PD098059 remain pluripotent, contributing to

differentiated tissues including the germ cells in chimaeric mice [Burdon et al., 1999]. Propagation of pluripotent ES cells therefore contrasts with the situation observed in many somatic cell types, where stimulation of the Ras/ERK pathway is required for progression through the G1/S phase of the cell cycle [Pages et al., 1993]. ES cells, however, in common with epiblast cells in postimplantation embryos, have an unconventional cell cycle [Mac Auley et al., 1993; Savatier et al., 1994]. They proliferate rapidly with a doubling time of 8–12 h and have a shortened G1 phase. Dephosphorylation of the retinoblastoma protein, an event normally required for entry into G1, is not detected in ES cells progressing from M to S phase [Savatier et al., 1994]. Undifferentiated ES cells contain low levels of the G1 cyclins D and E [Savatier et al., 1996]. Furthermore, their proliferation is unaffected by overexpression of $p16^{Ink4a}$, a specific inhibitor of G1 cyclin dependent kinases [Savatier et al., 1996]. ES cells therefore lack the control mechanisms that regulate the G1/S transition in somatic cells. This may provide a rationale for the lack of a requirement for ERK signaling in ES cell proliferation.

Role of SHP-2/ERK Signaling in ES Cell Differentiation

In contrast to its apparent redundancy in self-renewal signaling, SHP-2 may play an important role in regulating the differentiation of ES cells. Overexpression of catalytically inactive SHP-2 proteins in ES cells inhibits their differentiation both in monolayer culture and in embryoid bodies [Burdon et al., 1999; Burdon, unpubl. data]. Furthermore, $SHP\text{-}2^{\Delta 46-110}$ ES cells are compromised in their capacity to differentiate upon withdrawal of LIF, or when aggregated to form embryoid bodies [Qu et al., 1997; Qu and Feng, 1998].

Mouse embryos homozygous for either the *SHP-2*$^{\Delta 46-110}$ mutation, or an independently generated null mutation of SHP-2 die between embryonic day 8.5 and 10.5 [Arrandale et al., 1996; Saxton et al., 1997]. Examination of the *SHP-2*$^{\Delta 46-110}$ embryos revealed that they fail to gastrulate properly and exhibit multiple defects in the generation of both embryonic and extra-embryonic mesoderm. Interestingly, injection of mRNA encoding catalytically inactive SHP-2 into *Xenopus* oocytes also disrupts gastrulation and the formation of mesodermal structures [Tang et al., 1995]. Taken together these observations point to a role for SHP-2 as a critical regulator of early differentiation processes in the embryo.

Several independent lines of evidence indicate that ERK activation is an important effector of differentiation in the early embryo. Mesoderm formation in *Xenopus* is blocked both by over-expression of negative regulators such as the phosphatase MKP-1 and inhibitory mutant components of the Ras/ERK pathway [Whitman and Melton, 1992; LaBonne et al., 1995]. Significantly, overexpression of a constitutively activated member of this pathway is sufficient to induce de novo expression of mesodermal markers [LaBonne et al., 1995]. Interestingly, ES cells expressing the constitutively activated H-Ras (G12V) mutant exhibit an increased propensity to differentiate [Cheng et al., 1998]. In mouse, elimination of Grb2, an adapter protein downstream of SHP-2 and upstream of Ras, prevents egg cylinder development, but apparently does not impair self-renewal of ES cells [Cheng et al., 1998]. Furthermore, mutation of the gene encoding a transcription factor regulated by the Ras/MAPK signaling pathway, serum response factor, disrupts gastrulation and formation of mesoderm in mouse embryos [Arsenian et al., 1998]. Again, however, loss of serum response factor activity also appears not to compromise the propagation of ES cells.

The exact stage(s) at which SHP-2 or ERK signaling is required for the process of ES cell commitment and differentiation is not known. Differentiation of ES cells in vitro, through the formation of embryoid bodies, is associated with an induction of expression of G1 cyclins, a lengthening of the G1 phase and a decrease in the rate of cell division [Savatier et al., 1996]. This transition, probably reflects some of the changes that normally occur within the embryo at gastrulation [Wianny et al., 1998]. The differentiation of epiblast cells may therefore represent a point at which pluripotent stem cells become sensitive to G1-dependent growth regulatory mechanisms. ERK activity may be required in order to establish the regulated G1 phase by inducing expression of cyclin D1, and for cell cycle progression of cells that enter this state. ERKs probably also contribute to expression of differentiation genes.

STAT3 and Regulation of ES Cell Pluripotency

On the basis of the studies described above, it is evident that STAT3 plays a key role in mediating gp130-dependent self-renewal signals in ES cells. In fact, the absence of a requirement for the other main class of signals stimulated via gp130, the ERKs, points to the possibility that STAT3 activity alone may be sufficient for

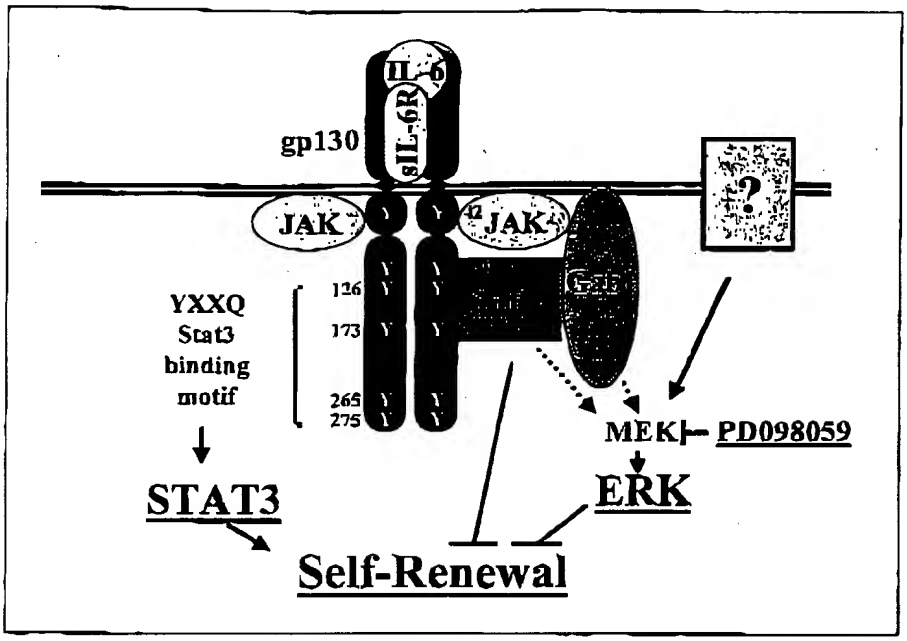

Fig. 6. Regulation of ES cell self-renewal by the STAT3 and ERK signaling pathways.

blocking the differentiation of ES cells. The development of constitutively activated or conditionally regulated forms of STAT3 will allow this hypothesis to be tested directly. In order to make this proposition unassailable, it will be necessary to evaluate the pluripotency of ES cells, not only on the basis of morphology or expression of stem cell markers but also by testing the capacity of the cells to undergo multilineage differentiation in chimaeric embryos. These qualifications, notwithstanding, it is clear that STAT3, either alone or in conjunction with other signal transducers, directs gene expression necessary for maintaining the pluripotent phenotype of ES cells.

How does STAT3 activity block the differentiation of ES cells? One possibility is that STAT3 maintains the expression of specific genes that define the pluripotent phenotype. A potential candidate might be the transcription factor Oct4. This regulator has been shown to be absolutely required for establishing and maintaining pluripotent stem cells in the embryo [Nichols et al., 1998]. Understanding the nature of the relationship between STAT3 and Oct4 is therefore an important issue. However, whereas the complete absence of stem cells in *oct4*$^{-/-}$ embryos indicates that Oct4 has a fundamental role in maintaining pluripotency, the formation of the epiblast in *STAT3*$^{-/-}$embryos indicates that the function of STAT3 is dispensable, and therefore that any Oct4/STAT3 interaction is not obligatory in vivo.

STAT3 has also been implicated in regulating the expression of genes involved in growth control. Activation of STAT3 via gp130 is associated with altered expression of Fos, Myc, Jun, and Bcl2 [Hirano et al., 1997]. It has also been shown that STAT3 enhances Src-dependent transformation of fibroblasts [Bromberg et al., 1998; Turkson et al., 1998]. Both DNA binding activity and serine phosphorylation of STAT3 are necessary, indicating that the transcriptional activity of STAT3 is required for mediating cell transformation.

STAT3 might maintain the unusual cell cycle of ES cells. Results from studies of STAT6 null cells and gp130 signaling in BAF3 cells demonstrate that STAT activity is associated with reduced expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, and thereby promotes cell cycle progression. Interestingly, overexpression of cyclin A, a known target of $p27^{Kip1}$, is reported to increase the rate of transit through G1 in somatic cells [Resnitzky et al., 1995]. How directly STAT activity is involved in suppression of $p27^{Kip1}$ transcription is unclear, but a potential role for the inhibitory STATs, such as STAT3β, is intriguing.

In addition to possible roles in controlling gene expression and promoting cell cycle progression, STAT3 might function to block the actions of signal transducers that direct differentiation. Examination of gp130 signaling in several different cell types indicates that STAT3 and ERK signaling can be mutually antagonistic [Fukada et al.,

1996; Bonni et al., 1997]. Given the evidence that ERK activation is likely to play a role in the establishment of differentiated cell lineages, the role of STAT3 in ES cells may include blocking the actions of this signal (fig. 6). This could be effected at the level of activation, or STAT3 and ERK signaling pathways may converge and compete at the level of gene expression. STAT3 may act by repressing transcription of differentiation genes, whereas ERK-dependent factors may induce their expression.

Further investigations into how STAT3 and ERK signals regulate ES cell self-renewal are likely to provide valuable insights into understanding the regulation of stem cell pluripotency. On the basis of current evidence it appears that the balance between these two pathways may determine the choice between self-renewal and differentiation (fig. 6). Importantly, therefore, selective inhibition or activation of these signals and their targets may allow greater control over the propagation, manipulation and differentiation of ES cells.

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